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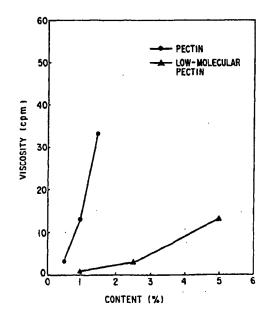
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# (54) Low-molecular pectin, and food and drink which contain low-molecular pectin

- (57) A novel pectinase for degradation a pectin or pectic acid is disclosed wherein
  - (1) the novel pectinase is an endopolygalacturonase produced from a genus Saccharomyces,
  - (2) the optimal pH is 4.0,
  - (3) the stable pH range is 4.0 to 8.0,
  - (4) the optimal temperature is 45°C,
  - (5) the enzymatic activity is stable up to 45°C, and
  - (6) the molecular weight is 38,000. A low-molecular pectin having a low viscosity and a high solubility and maintaining the physiological activity as the dietary fiber, and food and drink each of which contains 0.01 to 50 wt% of the low molecular pectin are also disclosed.



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#### Description

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The present invention relates to a low-molecular pectin converted from a pectin while the physiological activity of the pectin as a dietary fiber is maintained, and food and drink which contain the low-molecular pectin.

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Dietary fibers are defined as hard digestive components in foodstuffs which cannot be digested by human digestive enzymes. The dietary fibers include non-digestive organic materials such as chitin and chitosan in addition to plant cell wall components such as cellulose, lignin, and pectin. In recent years, these dietary fibers are found to have various activities such as a defecation improving effect and an activity of reducing the cholesterol content of blood and to play an important role in preventing diseases of adult people.

Of these dietary fibers, pectic substances such as a pectin and pectic acid have a strong activity as the dietary fibers. Various effects such as a defecation improving effect, an effect of repressing the level of the cholesterol content of blood, an effect of repressing formation of gallstones, and a hypertensive repression effect have been reported. Conventionally, pectic substances have been used as stabilizers in jams, fruit jellies, yoghurt drinks, and lactic acid beverages in food industries. Since the pectic substances have the above effects, they are expected as dietary fibers to be added in food and drink.

A pectic substance is bound with the cellulose in an unfipe fruit or plant to be present in the form of a complex called a protopectin. In particular, the protopectin is contained in citrus fruits, apples, and chinese quinces in large amounts. Although this protopectin is insoluble, it is hydrolyzed to produce a soluble pectin or pectic acid when the fruit is ripened.

Of these products, the pectin is a polysaccharide containing galacturonane as a polymer of galacturonic acid as a major component and small amounts of rhamnose, arabinose, xylose, and galactose and having a molecular weigh of 200,000 or more.

The pectin generally has a low solubility and a high viscosity and tends to gel. For this reason, although the pectin has the various effects as described above; only a small amount of pectin is added to food and drink, and it is difficult to add the pectin in food and drink in an amount enough to expect the activity of the dietary fiber.

It is, therefore, the first object of the present invention to provide a low-molecular pectin which has a high solubility and a low viscosity and maintains the physiological activity as the dietary fiber.

It is the second object of the present invention to provide food and drink which contain the low-molecular pectin.

In order to achieve the above objects of the present invention, a pectin is degraded using a pectinase to obtain a low-molecular pectin which has a low viscosity and a high solubility. The present inventors made extensive studies on many pectinase on the basis of the above assumption. As a result, the present inventors found that endopolygalacturonases (EC3. 2. 1. 15) derived from a yeast (i.e., Kluyveromyces fragilis, JTF-1) belonging to the genus Kluyveromyces, a yeast (i.e., Geotrichum candidum, JTF-2) belonging to the genus Geotrichum; a yeast (i.e., Candida Kefyr, JTF-3) belonging to the genus Candida, and a yeast (i.e., Saccharomyces bayanus, JTF-4) belonging to the genus Saccharomyces were suitable as pectinase. In addition, the present inventors also found that even if enzymes obtained from the above yeasts were caused to act up to the degradation limit, the decrease in molecular weight of the pectin by degradation was stopped at the molecular weight of about 20,000; and degradation no longer progressed. The present inventors also found that low-molecular pectins having molecular weights of 20,000 could be obtained by appropriate reaction condition control.

The present inventors have deposited the micro-organisms for producing the pectinase (endopolygalacturonases) used in the present invention designated as JTF-1 (accession number: FERM BP-4056) on October 11, 1991, JTF-2 (accession number: FERM BP-4057) on December 19,1991, JTF-3 (accession number: FERM BP-4058) on March 6, 1992, and JTF-4 (accession number: FERM BP-3916) on July 9, 1992 with the Fermentation Research Institute, Agency of Industrial Science and Technology located at 1-3, Higashi 1-chome, Tukuba-shi, Ibaraki-ken 305, Japan in accordance with the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedures.

The present inventors found for the first time that JTF-4 produced an endopolygalacturonase.

Furthermore, a novel pectinase having the following natures (i) to (vi) is provided:

- (i) The novel pectinase is an endopolygalacturonase which is produced from the genus Saccharomyces and degrades the pectin and the pectic acid.
- (ii) The optimal pH upon reaction at 35°C for 20 minutes is 4.0.
- (iii) The stable pH range upon heating at 35°C for 60 minutes is 4.0 to 8.0.
- (iv) The optimal temperature upon reaction at a pH of 5.0 is 45°C.
- (v) The enzymatic activity upon heating at a pH of 5.0 for 60 minutes is stable up to 45°C.
- (vi) The molecular weight is 38,000.

According to the first aspect of the present invention, the endopolygalacturonases (the endopolygalacturonases produced from the yeasts JTF-1 to JTF-4 will be referred to as JTFP-1, JTFP-2, JTFP-3, and JTFP-4, respectively) pro-

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duced from JTF-1, JTF-2, JTF-3, and JTF-4 are caused to act on pectins to obtain low-molecular pectins. According to the second aspect of the present invention, there are provided food and drink which contain 0.01 to 50 wt% of the low-molecular pectin. Fire the grant time of This invention can be more fully understood from the following detailed description when taken in conjunction with The end of the following manager of the second the accompanying drawings, in which: eus de**ol**ităția di se il nem la cele a 30 € 3 The Control of Expression in the State of the Control of the Contr Fig. 1 is a graph showing the relative activity and the pH to determine an optimal pH of an enzyme (JTER-4); Fig. 2 is a graph showing the relative activity and the pH to determine a stable pH range of the enzyme (JTFP-4); Fig. 3 is a graph showing the relative activity and the temperature to determine an optimal temperature of the enzyme (JTFP-4); Fig. 4 (18 576) box, odo-rijbna laderiji ba buur se moredus odogo iji - miliji a kilaseriy po Fig. 4 is a graph showing the relative activity and the temperature to determine a stable temperature range of the enzyme (JTFP-4); and the Matter Selection of the Control of the Co 500 YU (17 6125/10 3 Fig. 5 is a graph showing a viscosity curve of a low-molecular pectin according to the present invention, Annual of and some of the office of the perite substances and the above effects that are expected as a contract of The present invention will be described in detail below. 15 At of the moderning An endopolygalacturonase (UTFP=4) derived from JTF-4 belonging the genus Saccharomyces according to the present invention will be described below-costs and to the control of the costs of JTFP-4 as the enzyme of the present invention acts on a pectin and pectic acid to hydrolyze them. JTFR-4 has the following physicochemical properties: 30 600 auto, aspire and society as long a seege to a machine tufficem is the some electroscop bases as the second of commercing again (1) Substrate Specificity sit and tends to get For this reason, afting the co the permit products to the schooling area of the JTFP-4 according to the present invention degrades the pectin and pectic acid, but does not degrade soluble starch, dextrin, and xylan, visitable and to yilliams and that page on poor thromal is noticed an object to the program of the this interesting and characteristic parties and a solution of the property of this allow viscosity and organizative placehological activity as the detary fiber. (2) Optimal pH it is the second cuted of the present intention to group food and drink which contain the low-molecular, secon TTP-4 according to the present invention has an optimal phoneana pH of 4 to expose entremy of rebrond to selection with a selection and a recommendation of the present investment of the selection of the selecti (3) Stable pH Rangert is a contravert eaeric, and thought as a resolution as a process of the earliest of the tiases (EO2 Committee of the pression of the Nurveron ces tradition of the control to the contr JTFP-4 according to the present invention is stable in the pHigangeof 4 to 8. missing in the present invention is stable in the pHigangeof 4 to 8. missing in the present invention is stable in the pHigangeof 4 to 8. missing in the present invention is stable in the pHigangeof 4 to 8. missing in the present invention is stable in the pHigangeof 4 to 8. missing in the philadelphia in t บยใด าging to the ge <u>in Candida, and aly</u>east (i.e., <u>Saccharomyces bayanus,</u> JT∺-4) belonging to the <u>genus S</u>e <u>ชอา กอ</u> ences were suitable as pectinase, in addition, the present inventors also found that even if end viriginal addition, the present inventors also found that even if end viriginal addition, the present inventors also found that ablive yeasts wall, a lised to act up to the degradation limit, the decrease in motechian weight  $\delta$  the preci The enzymatic activity of JTFP 4 is 38.9 trifts/mg protein, () contract of enzyme for producing deumolof a reducing group of the hydrolysate per minute at 35°C in the hydrolysis of pectic acid) as consecutive or contest at 35°C in the hydrolysis of pectic acid) as consecutive or contest at 35°C in the hydrolysis of pectic acid) as consecutive or contest at 35°C in the hydrolysis of pectic acid) as consecutive or contest at 35°C in the hydrolysis of pectic acid) as consecutive or contest at 35°C in the hydrolysis of pectic acid) as consecutive or contest at 35°C in the hydrolysis of pectic acid) as consecutive or contest at 35°C in the hydrolysis of pectic acid) as consecutive or contest at 35°C in the hydrolysis of pectic acid) as consecutive or contest at 35°C in the hydrolysis of pectic acid) as consecutive or contest at 35°C in the hydrolysis of pectic acid (acid ) as consecutive or contest at 35°C in the hydrolysis of pectic acid (acid ) as consecutive or contest at 35°C in the hydrolysis of pectic acid (acid ) as consecutive or contest at 35°C in the acid (acid ) ac appropriate elegan la considera control. (5) Optimal Temperature 1980 1056, and price of screening operation and the accept about the company of the service of the company of the com sed in the pursery of the designation as ATF-4 (accussion number FERBABEA BEACH FIRE 40 JTFP-4 according to the présent invention has an optimal temperature near 45°C. The High has reported not 3980000 resol, and cliest is been on member. FBFMC EP-3916, on John 3, 1896 will be referenced to (6) Stable Temperature in the control of accounts at an expense to be about vigorous from the account of the graph of considered to the control of the aty on the International Necognition of the Cerus in the Court JTFP-4 according to the present invention is stable until 45°C. 45 The second of the first of the party of the second and the second of the (7) Influence of Metal Ion and Inhibitor ( ) Six 20 ( ) 13 20 to grow 42 and notice of the contract of the con JTFP-4 according to the present invention is inhibited by 69% with barium chloride, but is not inhibited with magnesium sulfate. Carried Services Comment (8) Molecular Weight The molecular weight of JTFP-4 is 38,000. 55 (9) Amino Acid Composition JTFP-4 according to the present invention has a maximum content of glutamine and glutamic acid in a molecule

(130 residues per molecule).

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According to the present invention, the endopolygalacturonase is caused to act on the pectin to obtain a low-molecular pectin.

The endopolygalacturonases generally exist in bacteria, yeasts, fungi, and higher plants. Many steps are required to purify the enzyme from these sources. That is, cells are removed from a culture solution containing microorganisms or the like to obtain a culture supernatant. The culture supernatant is subjected to ammonium sulfate-precipitation to salt out only a protein. The protein is separated based on charges thereof using an ion exchange material. The enzyme is separated by gel filtration in accordance with molecular weights, thus purifying the endopolygalacturonase in accordance with such a general enzyme purification process.

According to the present invention, when a commercially available pectinase is used, purification must be performed to eliminate pectin esterase and hemicellulase from the pectinase.

Since JTFP-1, JTFP-2, JTFP-3, and JTFP-4 produced from JTF-1, JTF-2, JTF-3, and JTF-4, respectively, are extracellular enzymes secreted outside the microorganisms, the culture supernatant can be directly used as a crude enzyme solution. The culture supernatant can be generally obtained such that the yeast is cultured on an agar slant and is then cultured in mass production. The resultant cultured product is centrifuged to eliminate the microorganisms. In this manner, the culture supernatant obtained using the yeast can be directly used in an enzymatic reaction, thereby advantageously simplifying the enzyme purification process.

The culture supernatant is preferably subjected to a simple treatment such as dialysis, ultrafiltration, ion exchange, or gel filtration to eliminate the yeast smell produced in the reaction using this enzyme and to obtain a more transparent solution? As a second of the second of t

A low-molecular pectin is obtained such that the purified product, culture supernatant, or its treated product of the endopolygalacturonase obtained as described above is reacted with a suspension obtained by suspending a pectin in a buffer solution such as acetic acid.

The type of endopolygalacturonase used in the present invention is not limited to a specific one if it reacts with a pectin to produce a low-molecular pectin having a molecular weight of about 20,000 to 80,000. However, JTFP-1 to JTFP-4 free from laborious operations such as enzymatic purification are preferably used.

As a pectin used in the present invention, any pectin material can be used, and its origin is not limited to a specific one. Therefore, generally known pectins originating from fruits, such as a lemon pectin and an apple pectin can be used insertion in a lemon pectin and an apple pectin can be used insertion. If a long representation is to the lemon pectin and an apple pectin can be used insertion.

ি পাদিৰ reaction between the pectin and one of aTFP-1 to aTFP-4, a purified product, a culture supernatant (crude i enzyme solution), or its treated product may be used to react with the pectin. ভাষা বিভাগ নিয়ন বিভাগ

The degradation reaction by the enzyme is preferably performed for a reaction time of 12 to 48 hours when the content of the yeast culture supernatant is 5 to 20 parts by weight with respect to 1 part by weight of the pectin. The preferable reaction temperature and pH are those which allow a sufficient reaction and do not inactivate the endopolygalacturonase, i.e., 30 to 50°C and a pH of 4.0 to 8.0.

According to the present invention, evertifithe enzymatic reaction is performed at the degradation limit, the degradation of the pectin is stopped when its molecular weight is about 20,000. Therefore, by controlling the reaction conditions such as the reaction time, a low-molecular pectin having an arbitrary molecular weight falling within the range of about 20,000 to 80,000 can be obtained.

Although the low-molecular pectin according to the present invention can have a molecular weight of about 20,000 to 80,000, the molecular weight preferably falls within the range of about 50,000 to 70,000 in view of retention of the physiological activity as the dietary fiber and ease in addition of the low-molecular pectin in food and drink. A low-molecular pectin most preferably has a molecular weight of about 60,000.

The degraded product of the pectin may be directly dried and used, or may be further treated.

When a further treatment is to be performed, the degraded product of the pectin is purified by dialysis or ultrafiltration to eliminate galacturonic acid and its oligosaccharide in the degraded product and acetic acid used as the buffer solution in the reaction. The purified degraded product is precipitated using an organic solvent such as ethanol or acetone or dried by freeze drying or spray drying to obtain a powder for later applications.

According to the present invention, there are provided food and drink which contain low-molecular pectins of the present invention.

The low-molecular pectin obtained by the above method according to the present invention has a molecular weight falling within the range from that of a polysaccharide such as pectin or agarose to that of an oligosaccharide such as maltooligosaccharide or fructooligosaccharide. Although the low-molecular pectin has a lower viscosity and a higher solubility than those of the original pectin, it has a defecation improving effect as one of the physiological activities of the dietary fiber.

On the other hand, since the low-molecular pectin according to the present invention has the above properties, it can be contained in an amount which allows to maintain the physiological activity as the dietary fiber, i.e., 0.01 to 50 wt%, and preferably 0.1 to 5 wt%, which cannot be conventionally contained, in a variety of food and drink such as

juices, candies, breads, and jams.

The food and drink which contain low-molecular pectins according to the present invention exhibit improved physical properties and an improved palate at the above contents. These physical properties and palate are different from those obtained in a case wherein a conventional pectin is added to food and drink.

As described above, since the enzymes (JTEP-1 to\_JTEP-1) used in the present invention are extracellular enzymes secreted outside the microorganisms, the culture supernatant can be directly used as a crude enzyme solution and in the enzymatic reaction. Therefore, the enzyme canadvantageously simplify the enzyme purification process and easily degraded the pectin into a low-molecular pectin. When the enzymes used in the present invention are caused to act on pectins up to the degradation limit, the decrease in molecular weight of the pectin upon degradation can be stopped at about 20,000; and further degradation cannot progress according to the characteristic feature of the enzyme. By controlling the reaction conditions, a low-molecular pectin having a molecular weight falling within the range of about 20,000 to 80,000 can be obtained.

Since the resultant low-molecular pectin has allow viscosity and a high solubility and can maintain the physiological activity (e.g., a defecation improving effect) of the dietary fiber, the low-molecular pectin can be easily added in the food and drink in an amount enough to provide the physiological activity as the dietary fiber.

The present invention will be described by wayrof, its examples, but is not limited thereto, and the control of the present invention will be described by wayrof, its examples, but is not limited thereto, and the present invention will be described by wayrof, its examples, but is not limited thereto, and the present invention will be described by wayrof, its examples, but is not limited thereto, and the present invention will be described by wayrof, its examples, but is not limited thereto, and the present invention will be described by wayrof, its examples, but is not limited thereto, and the present invention will be described by wayrof, its examples, but is not limited the present invention will be described by wayrof, its examples, but is not limited the present invention will be described by wayrof, its examples, but is not limited the present invention will be described by wayrof, its examples, but is not limited the present invention will be described by wayrof, its examples, but it is not limited the present invention will be described by wayrof, its examples, but it is not limited to be described by wayrof, its examples, but it is not limited to be described by wayrof, its examples, but it is not limited to be described by wayrof, its examples, but it is not limited to be described by wayrof, its examples, but it is not limited to be described by the present in the pres

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Parts and percentage represent parts by weight and wt% throughout the examples, unless otherwise specified.

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Example 1 responsible one sare negresses and with some of every of levels and matrices are improperly

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Method of Culturing JTE-1 to JTE-4 and Preparation of Crude, Enzyme, Solutions, Inham you group to english of the court of

(1) Method of Culturing JTEst: and: Preparation of: Crude Enzyme. Solution as a notified on the decimal and the second of the control of the

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Kluyveromyces tragilis JTF-1 was cultured on the slant of potato sucrose agar (pH of 5,0) at 27°C for 34 hours. The cultured Kluyveromyces fragilis in one platinum loop was inoculated in 50 m² of a medium (pH of 5.0) containing 5% of glucose; 0.2% of ammonium phosphate, 0,1% of potassium primary phosphate; 0,1% of magnesium sulfate and 0.4% of a yeast extract and was stationarily cultured at 27°C for 3 days. This cultured product was inoculated in 1½ of a medium having the same compositions as above the culture medium and was stationarily cultured at 27°C for 3 days. The resultant cultured product was centrifuged at 13,000 rpm for 19 minutes to eliminate JTF-1, thereby obtaining a culture supernatant of the access results as wells notify each the same supernatant of the access results.

endopolygalacture has alle 160 to 50°C and a pH of 4.0 to 8.0.

According to the present invention, ereitable&eavysn3.gbur0.to:nottapage9TUrgnirutluO to botteM (S) and a conduction of the present invention.

A:culture supernatant was obtained following the same procedures as in (1) except that Geotrichum.cardidum JTF-2 was used in place of Kluyveromyces fragilis JTF-1.

bernet soled as a contraction of the same procedures as in (1) except that Geotrichum.cardidum JTF-2 was used in place of Kluyveromyces fragilis JTF-1.

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(3) Method of Culturing JTF-3 and Preparation of Crude Enzyme Solution desistant in the control of the control

Candida Kefyr JTF-3 was cultured on the slant of potato sucrose agar (pH of 5.0) at 22°C for 3 days. The cultured Candida Kefyr in one platinum loop was inoculated in 50 m² of a medium (pH of 5.0) containing 5% of glucose, 0.2% of ammonium phosphate, 0.1% of potassium primary phosphate, 0.1% of magnesium sulfate, and 0.4% of a yeast extract and was stationarily cultured at 22°C for 3 days. This cultured product was inoculated in 1² of a medium having the same composition as the above culture medium and was stationarily cultured at 22°C for 4 days. The resultant cultured product was centrifuged at 8,000 rpm for 10 minutes to eliminate JTF-3, thereby obtaining a culture supernatant.

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(4) Method of Culturing JTF-4 and Preparation of Crude Enzyme Solution

Saccharomyces bayanus JTF-4 was cultured on the slant of potato sucrose agar (pH of 5.0) at 28°C for 3 days. The cultured Saccharomyces bayanus in one platinum loop was inoculated in 50 m² of a liquid medium (5% of glucose, 0.2% of ammonium phosphate, 0.1% of potassium primary phosphate, 0.1% of magnesium sulfate, and 0.4% of a yeast extract; pH of 5.0) contained in a 200-m² Erlenmeyer flask and was stationarily cultured at 28°C for 3 days. This cultured product was inoculated in 1² of a medium having the same composition as the above culture medium and contained in a 3-² Erlenmeyer flask and was stationarily cultured at 28°C for 3 days. The resultant cultured product was centrifuged at 8,000 rpm for 10 minutes to eliminate JTF-4, thereby obtaining a culture supernatant.

# Example 2

## Method of Preparing JTFP-4

The culture supernatant obtained in Example 1 was filtered through a millipore filter (pore size: 0.45 µm) to perfectly eliminate JTF-4. The culture filtrate was dialyzed overnight in a 0.02 M acetic acid buffer solution (pH of 5.0) at 5°C. About 600 mℓ of the dialyzed culture supernatant were adsorbed in an ion exchange column (S-Sepharose) and were eluted in accordance with a density gradient method using an aqueous sodium chloride solution. Active fractions were collected, and gel filtration column chromatography (Sephadex G-75) was performed using a 0.02 M acetic acid buffer solution as an eluent. This chromatogram exhibited one highly active peak. The fractions corresponding to the highly active peak were collected and dialyzed overnight in distilled water at 5°C. The dialyzed product was condensed to 5 mℓ by gel filtration. About 1 mg of a purified enzyme was obtained as a protein from 600 mℓ of the culture supernatant.

The enzymatic activity (one unit) was determined by measuring the number of reducing groups in the hydrolysate obtained by the enzymatic reaction in accordance with the Somogyi-Nelson method. That is, one unit is an amount of enzyme for producing it µmol of the reducing groups of the hydrolysate per minute at 35°C (the number of produced reducing groups is figured out as an amount of galacturonic acid). As a result of this measurement, the enzymatic activity according to the present invention was found to be 33.9 units/mg protein.

When SDS polyacrylamide electrophoresis was performed using this sample, the sample was detected as a single band.

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HTILD The following experiment was performed to examine the properties of the enzyme (JTFR-4) of the present invention the region and experiment was performed to example a region of the present invention the region and the region of the present invention the region and the region of the region o

## (1) Substrate Specificity

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In order to examine the substrate specificity of the enzyme, reactivity between the enzyme and substrates shown in trable 14 was examined. AM G S a to vm S C D b, behas easy not be introduced order to across seeking and substrates shown

30 TWO Each substrate was added so that the final concentration of a 0.2 M acetic acid buffer solution (pH of 5.0) was set to be 0.2%. 0.1 ml of an enzyme solution was added to 0.15 ml of each resultant solution and was reacted therewith at 35°O for 20 minutes. The presence/absence of the substrate degradation activity was detected by measuring the number of reducing terminals for each substrate substrate degradation activity for each substrate is shown in Table 1. A mark of in-Table 1 represents a substrate degraded by the enzyme, and a mark x represents a substrate not degraded by the cenzyme. Saw of the accusance of the angument of the substrate at the accusance of the substrate of the subst

Table 1

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Proposition of a second completes a			
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As is apparent from Table 1, the enzyme of the present invention can degrade the pectin and pectic acid, but does not degrade a soluble starch, dextrin, and xylan.

#### (2) Optimal pH

0.02 ml of an enzyme solution were added to 0.23 ml of a McIlvaine buffer solution having a pH of 2 to 7 and containing pectic acid to obtain a final concentration of 0.2% and were reacted therewith at 35°C for 20 minutes. The activity was measured by a Somogyi-Nelson method. Enzymatic activity values were measured as relative activities when the

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maximum activity value was defined as 100%. As shown in Fig. 1, the relative activities obtained by the Somogyi-Nelson method were plotted as a function of the pH to obtain an optimal pH. As is apparent from Fig. 1, the optimal pH of the enzyme of the present invention was near 4.

#### (3) Stable pH Range

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Buffer solutions were a 0.2 M McIlvaine buffer solution (pH of 3 to 7) and a phosphoric acid buffer solution (pH of 7 to 10). The 4.9 of the control of the

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to 10 and were treated at 35% for an hour. 0.15 ml of a 0.5 M acetic acid buffer solution (pH of 5.0) were added to the treated solution to adjust the pH to 5.0. Pectic acid/was added to this solution so that the final concentration was adjusted to 0.2%, the resultant solution was reacted at 35°C for 20 minutes, and the activity was measured by the Somogyi-Nelson method. Enzymatic activity values were measured as relative activities when the maximum activity value was defined as 100%. As shown in Fig. 2, the relative activities obtained by the Somogyi-Nelson method were plotted as a function of the pH to obtain a stable pH range. As is apparent from Fig. 2, the enzyme of the present invention was stable within a pH range of 4 to 8 and to time a 1 to 100 and 100 an

### (4):Optimal Temperatures in is and eliginal aid great Semiobe quayrops and to the relief of the control of the

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0.02 ml of the enzyme of the present invention were added to 0.23 ml of a 0.2 M McIlvaine buffer solution (pH of 5.0) containing 0.2% of pectic acid and were reacted therewith at a temperature of 20°C to 80°C for 5 minutes, and the activity was measured by the Somogyi-Nelson method. Enzymatic activity values were measured as relative activities when the maximum activity value was defined as 100%. As shown in Fig. 3, the relative activities obtained by the Somogyi-Nelson method were plotted as a function of the temperature to obtain an optimal temperature. As is apparent from Fig. 3, the optimal temperature of the enzyme of the present invention was about 45°C.

#### (5) Stable Temperature Range

0.02 m/ of the enzyme of the present invention were added to 0.13 m/ of a 0.5 M McIlvaine buffer solution (pH of 5.0) and were heat-treated at a temperature of 20°C to 65°C for 60 minutes. After the reaction solution was cooled with ice, 0.1 m/ of a 0.5% aqueous pectic acid-solution was added to each treated solution and was reacted the rewith at 35°C for 20 minutes, and the activity was measured by the Somogyi-Nelson method. Enzymatic activity values were activities obtained by the Somogyi-Nelson method were plotted as a function of the present invention was 73% up to 45°C, but was reduced to about 20% at 65°C. The stable temperature range of this enzyme was limited up to 45°C.

(6) Influence of Metal Ion and Inhibitor	Faces 1
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Influences of a metal ion and an inhibitor on the enzyme of the present invention were examined.

Each metal ion and the inhibitor in Table 2 were added in 0.15 mℓ of a 0.2 M acetic acid buffer solution (pH of 5.0) containing 0.002 mℓ of the purified enzyme solution to obtain a concentration of 1 mM. Each solution was reacted at 35°C for 5 minutes, and 0.1 mℓ of a 0.5% aqueous pectic acid-solution was added thereto. The resultant solution was reacted at 35°C for 20 minutes, and an inhibition ratio was calculated using the Somogyi-Nelson method. Results are shown in Table 2. The inhibition ratio is a relative value with reference to a case (0%) in which a metal or inhibitor is not added.

	•		
٠.	Table	2	

Influence of Metal and Inhibitor						
Compound	Concentration (mM)	Inhibition Ratio (%)				
No additive	•	0				
BaCl <sub>2</sub>	1	69				
KCℓ	. 1	35				
Pb(CH <sub>3</sub> COO) <sub>2</sub>	1	54				

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Table 2 (continued)

Influence of Metal and Inhibitor					
Compound Concentration (mM) Inhibition Ratio (%)					
MgSO <sub>4</sub>	1	0			
FeSO <sub>4</sub>	1	18			
CaC <sub>ℓ2</sub>	1	ртен тр. <b>31</b> д. (			

As is apparent from Table 2, this enzyme was most inhibited (74%) by EDTA. The enzyme was inhibited by a barium ion (barium chloride) by 69%. No inhibition was found with a magnesium ion (magnesium sulfate).

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# (7) Molecular Weight

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The molecular weight of this enzyme obtained in Example 2 was measured by SDS polyagrylamide electrophoresis to be 38,000.

#### (8) Amino Acid Composition

as recognition, in the following objects and the

The enzyme of the present invention was hydrolyzed with 6M hydrochloric; acid at 105°C for 24 hours. The hydrolyzed was analyzed by an amino acid analyzer (Hitachi, Model 835) to measure the amount of constituent amino acid. The measurement was repeated three times, and a ratio of the amino acid contents was calculated to obtain an amino acid composition. Results are shown in Table 3.

cuau saw fie gmação o (8) mile nos comerces. Table 3

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·	"Amino Asid C	omposition /d arms the co	nsk Bingernio in bivilistra
30 englispenito at the Micleave (f) o	36 (enul) Amino Acid	Amino Acid Residue (per molecule)	Loeron kali ong ATT (A) milandok kalika i ob
	Asparagine + aspartic acid	15	
	Threonine	ntage <sup>©</sup> 151.0 401	A-would because a description for the
<b>35</b> നൂ (ന്) ctriam ബോണ . മൂന്ന് വർത് ഒ	ti <b>Serine</b> pajaus enew (s to (A)	ស្ស៊ុ សមិនជាធំ <b>43</b> ) នាជនជប្រធ	193 <b>9</b> -071-4-04 (17450) - 5
	Glutamine + glutamic acid	130	,
	Glycine	<b>37</b> ක්දුමේ විය	Loanstate (clare tropics of
<b>40</b> 195 MR 0004 E - ม. 1 ฮ ตุดเลย ฮ ส. มาลังกระยาสตากง (b โดยปาละสตาติ		17 ឯកនយុវមានមនុស្ស មានបានប្រទេស មានប្រទេស	tis mika Napis an in kan
	Methionine	1	: *
	Isoleucine Hand of	ruser school ( <mark>6</mark> maarica (d.)	y et le mai i i i i i tha
45	Leucine	. 7	
nt avy smaly DH in Alv DH is very Alv Homen for 1000 His in	Tyrosine  Phenylalanine	la Brancis and Grand Brancis Learning and Carlos and Carlos La Brancis and Alexandra and	AMARIAN SANTANIAN SANTANIANI SANTANIAN SANTANI
	Lysine	8	
50	Histidine	6	· · · · · · · · · · · · · · · · · · ·
The State of the American Con-	Arginine	3	
	Proline	8	
55	Note) No experiments for detection tophan and cystine.	were performed for tryp-	

		EP	0 868 854	A2			
٠	acid and is the second larg	ble 3, the amount of amin		ie per molecule	is largest in the	e glutamine + gluta	mic
5	Example 4		1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	11.7		•	
	Preparation and Analysis of	f Lemon Low-Molecular F	Pectin				
	a) Preparation of Lemon Lo	ow-Molecular Pectin	-				
10			- while i	1984 8 12 2 1 1			
15	of 4.8), and 1 <i>t</i> of the cultur reacted at 40°C for 24 hou dialyzed overnight with respected to obtain 58:34	rs. The resultant reaction pect to 100-fold deionized	n (1) of Exam solution was water of the s	nple 1 was adde condensed by sample solution.	ed thereto. The a rotary evapo . In addition, the	resultant solution v rator at 60°C and v e dialyzed product v	vas vas vas
	(2) Preparation of Low-Mol	ecular Pectin by JTF-2 Cu	alture Supern	atant	MO1. 13	es Libroa bronsa - I	٠
20 ,	culture supernatant prepare	molecular pectin were obt ed in (2) of Example d war one-ed an anana of 6083	s used. ः / ✓	rket an tailer	cedures as in (	1) except that 1ℓ of	the :
,÷	(3) Preparation of Low-Mol			atant 39077-49	n, manyagan sua	villteastrik massimilisiri	Ť
<b>25</b>	60.74g of a low-molecu supernatant prepared in (3)	ılar pectin were obtained fo of Example 1 was used.				Figure 12 of the culti	
	(4) Preparation of Low-Mol	ecular Pectin by JTF-4 Cu	ilture Supern	atant	· • • • • • • • • • • • • • • • • • • •		
<b>30</b>	70.40g of a low-molecu supernatant prepared in (4)	lar pectin were obtained to of Example 1 was used.	ollowing the s	ame procedure	s as in (1) excep	ot that 11 of the culti	ure 3
	b) Analysis of Lemon Low-	i Molecular Pectin	basam	ecsa + erigaveo			
35~		lar pectins obtained in (1)	) to (4) of a) :	ennne Bonnes subjecteds	•	manageramanta (1)	٠ - ١
JJ -	(4).	gyr	1	nsteig – enmete Disteig	-	measurements (1)	) <b>(O</b> %
	(1) Measurement of Molecu	-	; ( ;	3000	do		
40	The main peak of each filtration column to calculat sample.	lemon low-molecular pec te its molecular weight us			analysis using a -82, Showa De		
	(2) Measurement of Ratio	of Galacturonic Acid to No	utral Sugar	inal la	J3		

(2) Measurement of Ratio of Galacturonic Acid to Neutral Sugar

After each lemon low-molecular pectin was perfectly decomposed using Driselase (KYOWA HAKKO), the ratio of galacturonic acid to neutral sugar was measured by HPLC analysis using a Shodex Sugar SH-1821 column (S. Matsuhashi, S. Inoue and C. Hatanaka, Biosci. Biotech. Biochem., 56, p. 1053 (1992)).

(3) Measurement I of Viscosity

A 5% solution of each lemon low-molecular pectin according to the present invention was prepared, and its viscosity was measured using an E type viscometer (Tokyo Keiki, VISCONIC ED Type).

Results in the above measurements (1) to (3) are shown in Table 4.

Table 4

	- 1.3 	Low-Molecular Pec- tin by JTF-1 Culture Supernatant	Low-Molecular Pec- tin by JTF-2 Culture Supernatant	Low-Molecular Pectin by JTF-3 Culture Supernatant	Low-Molecular Pectin
			59.25		70.40 6.6 × 10 <sup>4</sup>
	Molecular Weight	6.6 × 10 <sup>4</sup> · 441			19 0.0 × 10 10 11
	Galacturonic Acid : Neutral Sugar	87.7 : 12.4	87.1 : 12.9	911	86.5 : 13.5
	Viscosity (cp)	15.97	15.97	15.97	15.97
	Outer Appearance of	yellowish brown	yellowish brown	yellowish brown	yellowish brown
ļ	5% Aqueous Solution Section Se	n to elik kilone deleti ine	erloott themidale	taeq of usia new cent	

#### (4) Measurement II of Viscosity

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The Strate \$2.0 and the Land of the period of the lemon low-molecular pectin obtained in (f) of a) was compared with that of a lemon pectin. The viscosities were measured using an E type viscometer (50 rpm). Results are shown in Fig. 5: The viscosity of the pectin was considerably reduced. Similar results were obtained for other low-molecular pectins and periods and the pectin was considerably reduced.

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#### (5) Defecation Improving Effect

4-week old:SD male rats were fed with a commercial solid feed (oriental yeast solid feed MF) for 4 days and were divided into four groups each consisting of five rats. A feed containing the low-molecular pectin obtained in (1) of a) and components shown in Table 5, and a solid feed were supplied to each group, and the rats were fed for 9 days. The feces of the rats on the ninth day were collected. Results are shown in Table 6. The hardness of the feces by the solid feed was used as a reference. The hard feces are a (negative), and the soft feces are a (positive).

#### no grund with that deanly of by Results are summarized in Table 8 below: **C eldaT**

Component	Control Group (g)	Pectin Group (g)	Low-Molecular Pectin Group (g)
Casein	22	22	22
Lard 00	9	9 "2	9-3
Corn oil	1	1 (1)	: <sup>(c)</sup> : 1
Mixed Salt	3.5	brios 34 <b>3.5</b> ° -silure	3.5
Mixed vitamin	1	1 *	1 <b>1</b>
Choline chloride	0.2	0.2	0.2
Cholesterol	1	<sup>٧</sup> 9	1
Bile acid	0.25	0.25	0.25
Pectin <sup>1</sup> )		5	•
Low-molecular pectin <sup>1</sup> )	-	• ·	5
Sucrose	63.3	58.3	58.3

<sup>1)</sup> The pectin and the low-molecular pectin were prepared from a lemon p ctin (Wako Junyaku Kogyo).

- Table 6

1. The second se	Solid Feed	Control Group	Pectin Group	Low-Molecular Pectin
Softening	0	The state of the s	+	+

Judging from the above results, the low-molecular pectin prepared using the yeast of the present invention has a feces softening effect and was found to have a defecation improving effect.

Similar results were also obtained for other low-molecular pectins.

Example 5

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Applications of Low-Molecular Pecting (1977)

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Applications using the low-molecular pectin obtained in (1) of Example 4 will be described in the following a) to c).

a) 30% Apple Juice

6 parts of 5-time condensed apple juice, 10 parts of granulated sugar, 0.2 parts of DL-malic acid, 0.02 parts of sodium citrate, and 83 parts of distilled water were mixed with the part of the low-molecular pectin to prepare a 30% apple juice containing 1 wt% of the low-molecular pectins. (inc. 02) retemption actific according to the low-molecular pectins.

The juice containing the low-molecular pectin exhibited smooth nector-like physical properties. years biscoptian

b) Hard candy

Accomposition material (Table 3) containing the part of the low-molecular spectin was used to prepare an apple type hard candy a the last of the containing the part of the materials and the property of the containing the part of the containing the containing the part of the containing the contain

Sugar, millet jelly, and water were mixed with each other, and the resultant mixture was heated to 119°C. The lowmolecular pectin dissolved in a small amount of water was added to the above mixture and was boiled down to 147°C.
Citric acid, spices, and a coloring agent were added to and mixed in the boiled down mixture. The resultant mixture was cooled and molded. As a control, an apple type hard candy obtained by adding 1 part of a pectin was prepared and was compared with the hard candy of b). Results are summarized in Table 8 below.

	5.91	מני:		
Nioleau at Peuri Group (ci	wa. [g] 200 DinibeS [	Scrittol Group (g) aida	ina:	osd(uo.)
	Components	(parts by weight)		
· · · · · ·	Sugar	e	60	7.6.1
	Millet jelly		40	0.110
j. ÿ	Apple juice (5-time conde	ensed)	2	# B 34 71
	Water		17.5	engiges a say the
· 1. 1	Citric acid	<u>s</u> 1)	1	ria ku area (j. )
	Spices		0.1	
	Coloring agent	appropriate	amount	
	Low-molecular pectin		1	

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Table 8

i		Outer Appearance	Taste	Total Evaluation
r .	1% low-molecular pectin-added candy	properly dispersed	'1	very good
	1% pectin added-candy	lump of powder; not properly dis- persed	too sour; strange taste	not satisfactory

When 1 wt% of pectin was added to the candy material, the pectin formed a lump of powder and could not be properly dispersed. However, when the low-molecular pectin was added in the same amount as that of the pectin, the low-molecular pectin could be properly dispersed and facilitated the preparation of candies. In addition, the pectin-added candy was too sour and had a strange taste. However, the low-molecular pectin-added candy tasted good.

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c) Bread in the Age is transportural and the state of the state in Eq. (c) the state of the stat

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Bread was prepared using a composition material shown in Table 9.

5.73.2.5 parts of the low-molecular pectin were dissolved in water in advance, and this aqueous solution was mixed in the material (Table 9) except for a dry yeast. The resultant mixture was charged in a bread case of a Sanyo bread maker (SPM-B1), and the dry yeast was added thereto. The mixture was kneaded and fermented to bake the bread. As a confirm, bread was baked using the material composition (Table 9) from which the low-molecular pectin was omitted.

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e to be well and tredfer ao ation (pH of 4 Pt 4 as a last copyacted vice and the measurement of the formation

		idoic 5		
	Enzyme Solution Curamed from JTF 6	Components (part by weight) hato		Complete of Low-Moreovier R
	in Example 4 охоцог этов pullure super-			
30	method of (2) of Example 1 against 2000	e Sugare distriction attance.	ua ert <b>14</b> भ जि.	THE POST OF LOW SHOWS IN THE
	viure superciate it prace each the method	อ ลูกรู้ ใด ละเล่าสุดเกลดเกล	v (은 사업 걸대) 라이	A TO JUST Aleader of mild buffer solution
	with elstyperparent praceings in the method philotics with the method	jew lejugelom s ban nijo	VI-TOLEGUELDE	i meduzeren". "Leonouett. Gr
		Skim milk	6.8	
	Enzyme Solution Obtained from JTP-4	e Supernatant of Cude <b>Shortening</b>	AuD griff) nito	ल had sation of Low-Molecular कि
35	in Example 4 except that a culture suncer-			
	mathed or (4) of Example 1 against 2007	e restant obtained rawe	va 51480 √0 m	mero gristasio (detech u mata
	durie suppressar or eparagin the method	as used in placerol the c	33 55 mg mg	auco nertico pico e na la Anigno di Silin. Ciltras reggi ed Tilli escriba e Elabera e
	·		<u> </u>	1

The organoleptic test results are shown in Table 10. Bread containing about 0.5 wt% of the low-molecular pectin and bread of the control were almost the same, but the low-molecular pectin-added bread was softer than the control.

Comparation of the comparation of the

,	Outer Appearance and Taste	Total Evaluation	
0.5% Low-Molecular Pectin-Added Bread	Slight yeast-like smell; softer than the control; uni- formly baked in brown	good	
 Control	pleasant smell of bread; uniformly baked in brown	good	

The above tests a) to c) were also conducted for the low-molecular pectins obtained in (2) to (4) of Example 4, and similar results were obtained.

E	хa	m	ol	e	6
-	Λu		•		v

Apple low-molecular pectins were prepared from an apple pectin (Wako Junyaku Kogyo) obtained following the same procedures as in (1) of Example 4, using the culture supernatants prepared in (1) and (3) of Example 1. Each of the resultant low-molecular pectins had a molecular weight of 6.6 × 10<sup>4</sup>.

Example 7

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Preparation of Low-Molecular Pectin by Culture Supernatant Obtained by Dialysis

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(1) Preparation of Low-Molecular Pectin: Using:Culture Supernatant of Grade Enzyme Solution Obtained from JTF-1

A low-molecular pectin-was prepared following the same procedures as in Example 4 except that a culture supernatant obtained by dialyzing overnight 1ℓ of the supernatant obtained in the method of (1) of Example 1 against 300ℓ of a 0.025 M acetic acid buffer solution (pH of 4.8) was used in place of the culture supernatant prepared in the method of (1) of Example 1. The resultant low-molecular pectin had a molecular weight of 6.6 × 10<sup>4</sup>.

Bread was to the lide of the property on the state of the state of

(2) Preparation of Low-Molecular Pectin Using Gulture Supernatant of Crude Enzyme, Solution Obtained from JTF-2

A low-molecular pectin was prepared following the same procedures as in Example 4 except that a culture supernatant obtained by dialyzing overnight 1/ of the supernatant obtained in the method of (2) of Example 1, against 300/ of a 0.025 M acetic acid buffer solution (pH of 4.8) was used in place of the culture supernatant prepared in the method of (2) of Example 1. The resultant low-molecular pectin had a molecular weight of 6.6 × 10<sup>4</sup>.

(3) Preparation of Low-Molecular Pectin Using Culture Supernation of Crude Enzyme Solution Obtained from JTF-3

A low-molecular pectin was prepared following the same procedures as in Example 4 except that a culture supernatant obtained by dialyzing overnight 1ℓ of the supernatant obtained in the method of (3) of Example 1 against 300ℓ of a 0.025 M acetic acid buffer solution (pH of 4.8) was used in place of the culture supernatant prepared in the method of (3) of Example 1. The resultant low-molecular pectin had a molecular weight of 6.6 × 10<sup>4</sup>.

(4) Preparation of Low-Molecular Pectin Using Culture Supernatant of Crude Enzyme Solution Obtained from JTF-4

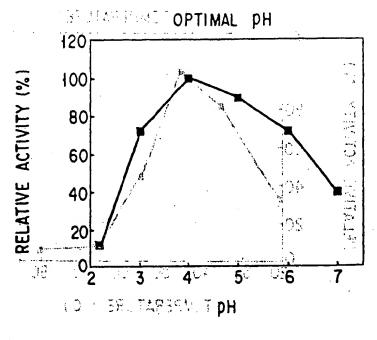
Skim milk

A low-molecular pectin was prepared following the same protectures as in Example 4 except that a culture supernatant obtained by dialyzing overnight 1/ of the supernatant obtained in the method of (4) of Example 1 against 300/ of a 0.025 M acetic acid buffer solution (pH of 4.8) was used in place of the culture supernatant prepared in the method of (4) of Example 1. The resultant low-molecular pectin had a molecular weight of 6.6 × 10<sup>4</sup>.

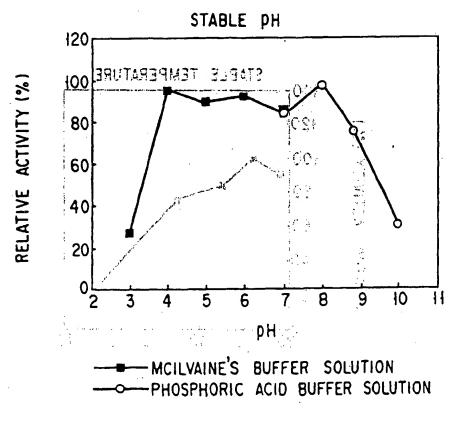
# Claims

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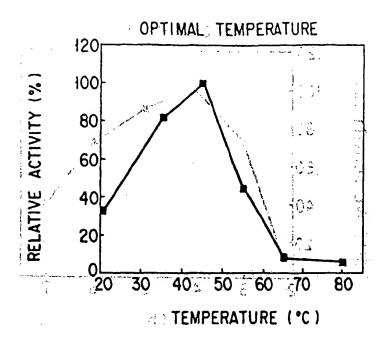
- 1. A low-molecular pectin obtained by causing an endopolygalacturonase to actions a pectin and a consequence of the consequence
- 2. A low-molecular pectin according to claim 1, characterized in that the endopolygalacturonase is produced from Kluyveromyces, a genus Geotricum, a genus Candida; or a genus Saccharomyces.
  - 3. A low-molecular pectin according to claim 1 or 2, characterized in that the pectin has a molecular weight of 6.6 x 10<sup>4</sup>.
  - 4. A food or drink characterized by containing the low-molecular pectin according to either one of claims 1 to 3, at 0.01 to 50 weight%.



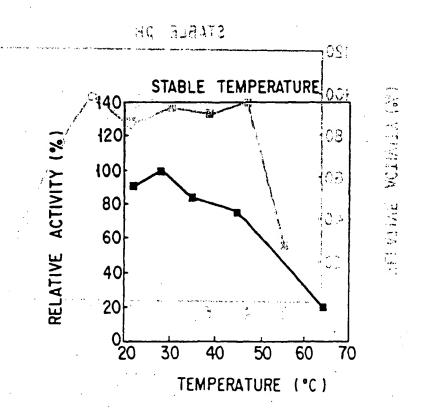
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F 1 G. 2



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F I G. 4

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